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14. ABSTRACT

The androgen receptor (AR) is a ligand-activated transcription factor that is essential for development and progression of prostate cancer. Using the N-terminus of AR as bait in the repressed transactivator yeast two-hybrid system, TAF1 was identified. TAF1 interacts with several proteins to promote or suppress gene transcription. In the present study, using GST pull-down assay we confirmed that TAF1 binds to NTD of AR through the E1/E2 and HAT domains. We demonstrated by co-immunoprecipitation and ChIP assays that TAF1 and AR bind in the nucleus and associate with an androgen response elements at the proximal/enhancer promoter of the prostate specific antigen (PSA) gene when the AR is transcriptionally active. In addition, TAF1 was shown in human prostate cancer tissue microarrays to steadily increase with duration of neoadjuvant androgen withdrawal and with progression to castration resistance. Furthermore, we found that TAF1 enhances AR transcriptional activity through its NTK and E1/E2 domains probably through ubiquitination of AR.

In conclusion, our results indicate that increased TAF1 expression is associated with progression of human prostate cancers to the castration-resistant state. Since TAF1 is a coactivator of AR that binds and enhances AR transcriptional activity, its overexpression could be part of a compensatory mechanism adapted by cancer cells to overcome reduced levels of circulating androgens.

15. SUBJECT TERMS
Androgen receptor, Prostate cancer, transcription

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Table of Contents

INTRODUCTION

Prostate cancer is the most commonly diagnosed non-skin cancer in men and one of the leading causes of cancer death. Androgen withdrawal therapies are still the most effective treatment for advanced disease, although it eventually progresses to the lethal castration-resistant stage. Evidence suggests that most cells in castration-resistant tumors retain androgen receptor (AR) that maintains its transcriptional activity through alternative pathways (1-7). The AR is a member of the steroid receptor family that shares common functional domains and structures (8, 9). This family of receptors has (i) a ligand-binding domain (LBD) located in the C-terminal region (ii) a hinge region (iii) a centrally located DNA binding domain (DBD), and (iv) an N-terminal domain (NTD). Between members of this family, the NTD domain has the highest degree of amino acid sequence variability, suggesting that this region has a major role in AR-specific transcription regulation (10-13). To identify novel NTD-interacting proteins, we employed the Tup1 repressed transactivator (RTA) yeast two-hybrid system (14) and TATA binding protein-Associated Factor 1 (TAF1) was identified as a previously unreported AR-interacting protein.

TAF1 is part of the TFIID complex (Transcription Factor IID), which consists of TATA binding protein (TBP) and approximately 15 TBP associated factors (TAFs). TAFs, including TAF1, mediate activator-dependent transcription in a promoter and tissue specific manner (15-17). The TAF1 gene contains 38 exons which span 98 kb of genomic DNA on chromosome X and encode an approximately 6 kb mRNA. TAF1 is a multifunctional protein that contains acetylation (HAT), ubiquitin activating/conjugating (E1/E2) and bipartite, kinase domains consisting of N- and C-terminal kinases (NTK and CTK, respectively). TAF1 is capable of autophosphorylation as well as specific phosphorylation of TFIIF (18), p53 (19), and Mdm2 proto-oncogene (20). TAF1 binds and modulates transcriptional activity of proteins, such as c-Jun (21), the Mdm2 (22), and cyclin D1 (23) that they can also influence AR activity and hence prostate cancer progression (24-26). The focus of this study is to identify if and how TAF1 modulates AR transcription, and to determine the role of TAF1 in prostate cancer progression. In particular, this research proposal will test the hypothesis that TAF1 directly modulates AR activity, and aids in the development and maintenance of castration-resistant prostate cancer. We proposed to test this hypothesis with the following four specific aims:

Specific Aim 1: To determine whether the TAF1/AR interaction specifically modulates AR transactivation

Specific Aim 2: To map functional interaction site(s) of AR and TAF1

Specific Aim 3: To determine the mechanisms through which TAF1 coactivates AR

Specific Aim 4: To assess the expression profile of TAF1 in prostate tumors:

a) To characterize TAF1 and AR expression at mRNA and protein levels in the *Pten*$^{-/-}$ prostate-specific knock out mouse model.

b) To determine the expression level of TAF1 in malignant versus benign human prostate tissues.

PROGRESS AND RESULTS

A. Summary of the first and second years report (specific aim 1, 2 and part of 3)

We have demonstrated that TAF1 interacted with AR within nucleus of LNCaP cells, a prostate cancer cell line that expresses AR. Using ChIP assay, we also showed that TAF1 associated with AR at the prostate specific antigen (PSA) promoter in the presence of hormone. To confirm the AR/TAF1 interaction and to determine the domains involved, GST pull-down assays were performed using GST-fusion protein with AR/NTD_{1-559}, $DBD_{541-665}$, or $LBD_{649-919}$ (Fig. 1). The results indicated that TAF1 interacts directly with AR and mapping the interaction domains of TAF1 and AR suggests that the HAT, E1/E2 and CTK domains of TAF1 are all involved in binding to AR. The pattern of binding of HAT and E1/E2 domains is similar to that seen with full length TAF1.

To determine the impact of AR-TAF1 interaction on AR transcriptional activity within cells, transactivation assays in prostate cancer cells were performed. TAF1 was either over-expressed (pHA-TAF1, kindly given by Dr. Tjian and Dr. Wong) or knocked down (siRNA duplexes; AAGACCCAAACAACCCCGCAT-3′and 5′-AACTACGACTACGCTCCACCA-3′) in prostate cancer cells and luciferase assays were performed. The results indicated that the expression level of TAF1 is directly correlated to AR activity only in the presence of hormone, when the receptor has been activated (Fig. 2). Since TAF1 is a member of the general transcription machinery complex it is expected that it influences on promoters of other genes. In our transactivation assays, we also found that the non-androgenic reporter (thymidine kinase-renilla (pRLtk-renilla)) is also modulated by TAF1 in a similar manner as androgen responsive reporters (Fig. 3A). To differentiate the effect of TAF1 on AR from its general effects on transcription and to determine which TAF1 domains are specifically involved in AR activation, we cloned various domains of TAF1 and repeated the luciferase assay in LNCaP cells. In contrast to full-length TAF1, TAF1 domains had no effect on the generic renilla construct with pRL-tk promoter, implying that general transcription is not affected. By comparison, while HAT and CTK domains had no significant effect on AR activity, NTK and the E1/E2 domains of TAF1 did enhance AR activity in a ligand dependent manner. NTK significantly enhanced AR transactivation by 2.4-fold, which is almost as much as the full-length TAF1. However, E1/E2 domain had even greater effect, enhancing AR activity over 3.4-fold (Fig. 3B).

Since NTK does not bind to AR and the CTK and HAT domains do not enhance AR transcriptional activity, we focused on the E1/E2 domain, which binds to AR and has the most profound effect on its transactivation. Ubiquitination is a posttranslational modification that mediates the covalent conjugation of ubiquitin to protein substrates. The functional role of ubiquitination was originally considered to be targeting proteins to the proteasome for degradation. However, it is now known that ubiquitination regulates many other processes in the cell, including membrane trafficking, DNA repair, and transcription (27). AR is also a direct target for mono and poly-ubiquitination (25, 28). To address whether TAF1 can ubiquitinate AR, LNCaP cells were co-transfected with pHis_6-Ubiquitin and either pHA-TAF1 or empty vector. Cells were then treated with 10% FBS RPMI followed by 6h treatment with vehicle or MG132, a proteasome inhibitor. The results indicate that TAF1 facilitates AR ubiquitination in a spectrum from mono to poly-ubiquitin form. To confirm the ubiquitination of AR by TAF1 and to assess

whether TAF1 is able to directly ubiquitinate AR, an in-tube ubiquitination assay was performed (29) and confirmed that TAF1 is able to directly ubiquitinate AR.

In 2009, we have tried to elaborate on the specific aim 3 and to address the specific aim 4. The results are discussed as follows:

B. Specific Aim 4: To determine the expression level of TAF1 in malignant versus benign human prostate tissues.

Although TAF1 is ubiquitously expressed in normal tissues (30) and also expressed in all prostate cancer cell lines including LNCaP and C4-2 cells (data not shown), analysis of expression in human tumours may be more relevant. Consequently, tissue microarray (TMA) of prostate biopsies from patients that had not received any neo-adjuvant hormone therapy was stained with antibodies that recognize human TAF1 (abcam, ab17360). Each array was prepared from 84 non-treated patients, with 4 cores per patients. Staining intensity was scored visually by a pathologist on a scale from 0 to 3, ranging from no staining (score 0) to very intense staining (score 3) (31, 32). The results indicate that there is no significant difference between benign and malignant human prostate samples when TMA is stained with TAF1 antibody (data not shown).

The expression profile of TAF1 was also assessed in patients who had undergone varying lengths of neo-adjuvant hormone therapy (NHT) prior to radical prostatectomy or autopsy, using NHT tissue microarrays (31, 33). Each NHT array is comprised of 336 tumor biopsies, which were obtained from triplicate cores of 112 tumors. Fig. 4A shows representative histology pictures of four test groups (<3 months NHT, 3-6 months NHT, >6 months NHT, and castration-resistant state) and Fig. 4B shows visual scoring analysis of the whole NHT array. Interestingly, we found the longer the NHT treatment, the higher the level of TAF1 protein. TAF1 expression of individual cores was compared between the different treatment groups and its level was found to be significantly higher in the 3-6 months NHT over the untreated group (Fig. 4B). Furthermore, there was an additional increase in TAF1 expression with longer NHT and with castration-resistant progression. Thus, increased levels of TAF1 expression are associated with progression to castration-resistant stage, and may have potential clinical value as a biomarker or a therapeutic target for advanced prostate cancer.

C. Specific Aim 3:

With the results that we have found, we were confident to submit our data in the Molecular Endocrinology journal. Upon requests of reviewers, we have performed the following experiments:

1- Co-immunoprecipitation assays in C4-2 cell line as a castration-resistant cell line.
2- ChIP assays in LNCaP cells when the AR is knocked down.
3- Ubiquitination assays in the presence and absence of androgen in LNCaP cells.
4- Ubiquitination assays with the E1/E2 domain of TAF1 as a comparison to the full-length TAF1.

We did not pursue the first part of the specific aim 4 (page 4) due to lack of time in breeding and selecting groups of *Pten*$^{-/-}$ prostate-specific knock out mice. Therefore, we have focused and elaborated on the specific aim 3, as per reviewers' request.

1- Co-immunoprecipitation assays in C4-2 cell line as a castration-resistant cell line.

LNCaP and C4-2 cells were respectively transfected with HA-TAF1 and treated with or without 1 nM of the synthetic androgen R1881. Using the Active Motif Co-IP kit, 1 mg of nuclear extracts of cells (calculated by the BCA assay) were subjected to IP with an anti-TAF1 antibody and analyzed by Western blot for TAF1 and AR (Fig. 5A, upper & lower panel, respectively). Upon IP of TAF1, the AR is co-immunoprecipitated with TAF1 in the presence of androgen in both cells, with the relatively stronger interaction within LNCaP cells (lane 8 compared to lane 12, lower panel). Together, these results indicate that TAF1 and AR are in a complex within LNCaP and C4-2 cells in the presence of androgen.

2- ChIP assays in LNCaP cells when the AR is knocked down.

We used ChIP assays to investigate whether TAF1 can bind to the promoter/enhancer of the PSA and whether this binding is through AR (Fig. 5B). LNCaP cells that stably express either DOX-inducible shRNA of the AR (LN-shRNA$_{AR}$) or DOX-inducible scrambled shRNA (LN-shRNA$_{SC}$) were used as described before (39). Cells were grown in CSS and treated with DOX for 48 h followed by 4 h treatment with or without R1881. DNA and proteins were then cross-linked, lysed, sonicated, and then immunoprecipitated with anti-TAF1 antibody (row 2), AR (row 3), or an equivalent amount of normal rabbit/mouse IgG as negative controls (rows 4 & 5 respectively). The DNA was purified and used as a template for PCR of the PSA proximal promoter (1st panel from left) and the enhancer (2nd panel). The result indicates that TAF1, like AR, binds with the PSA promoter/enhancer in the presence of androgen. This binding seems to be mainly AR-dependent since once the AR is knocked down, there is no detectable PCR product at the enhancer and just faint bands at the proximal promoter can be detected. To determine whether the binding of TAF1 to the PSA promoter is specific, the purified DNA obtained after TAF1 IP was subjected to PCR for a non-promoter region of the PSA promoter (3rd panel), as well as for β-actin promoter (4th panel). The absence of detectable PCR products in these regions indicate that TAF1 binding to the PSA promoter was promoter- and sequence-specific. Together, our ChIP assays results suggest that TAF1 and AR associate at the PSA promoter/enhancer once the AR is transcriptionally active.

3- Ubiquitination assays in the presence and absence of androgen in LNCaP cells.

To address whether TAF1 can ubiquitinate AR, LNCaP cells were cultured in 5% CSS media for 24 h and then co-transfected with pHis6-Ubiquitin and either pHA-TAF1 or empty vector. Cells were then treated with 5% CSS media with or without 1 nM R1881 followed by 6 h treatment with vehicle or MG132, a proteasome inhibitor. To show the His-ubiquitin conjugated status of AR in the presence and absence of MG132, after saving 5% input (Fig. 6A, lower panels), His-conjugated proteins were purified followed by a Western blot with antibody against AR. Fig. 6A (lanes 1 & 2) shows that in the absence of MG132 and hormone, there is no His-conjugated AR. However, very faint bands appear in the presence of hormone and are slightly higher with overexpression of TAF1 (lanes 4 versus 3). Lanes 5-8 show the same order of experiments in the presence of proteasome inhibitor. As expected, there is no ubiquitination of

AR in the absence of hormone while the total amount of poly-ubiquitinated AR is increased with MG132 and R1881 treatment when TAF1 is overexpressed (lanes 8 versus 7). Since Mdm2 (an E3 ligase) is involved in the poly-ubiquitination and consequently degradation of AR (25), we wanted to see if the Mdm2 protein could also be detected in this set of experiments. Hence, the same membrane was blotted with antibody against Mdm2. As shown in Fig. 6A (middle panels), the more ubiquitinated AR, the more Mdm2 within the protein complex. This suggests that through overexpression of TAF1 either the Mdm2 protein is also being ubiquitinated and targeted for degradation or TAF1 induces ubiquitination of AR through Mdm2.

4- Ubiquitination assays with the E1/E2 domain of TAF1 as a comparison to the full-length TAF1.

Given that the E1/E2 domain of TAF1 enhances AR activity, we wanted to know if this domain could also ubiquitinate AR in LNCaP cells under the same conditions as described above, except that the truncated E1/E2 of TAF1 was transfected instead of the full-length protein. Fig. 6B shows a substantial increase in the level of ubiquitinated AR once the E1/E2 is expressed in the presence of MG132 and R1881 (lane 4 versus 3). Together, these experiments indicate that TAF1 can ubiquitinate AR and its E1/E2 domain is sufficient to accomplish this.

KEY RESEARCH ACCOMPLISHMENTS

1. TAF1 interacts with AR within androgen-dependent and castration-resistant prostate cancer cells.
2. TAF1 associates at the PSA promoter/enhancer when the AR is transcriptionally active.
3. TAF1 binds to the N-terminal domain of AR. This interaction is mainly through HAT and E1/E2 domains of TAF1
4. The NTK and E1/E2 domains of TAF1 specifically enhance AR transcriptional activity and that mechanism is different from the role of TAF1 on general transcription machinery.
5. TAF1 through its E1/E2 domain can directly ubiqutinate AR in androgen-dependent manner.
6. TAF1 is a coactivator of AR that binds and differentially enhances AR transcriptional activity most likely through ubiquitination of AR.
7. The expression of TAF1 is increased during NHT therapy for advanced prostate cancer.

REPORTABLE OUTCOME

Publication:

1. **Tavassoli P.**, Wafa L. A., Cheng H., Zoubeidi A., Fazli L., Gleave M., Snoek R., Rennie P. S., TAF1 Differentially Enhances Androgen Receptor Transcriptional Activity via Its N-Terminal Kinase and Ubiquitin-Activating and -Conjugating Domains. *Mol Endocrinol. 2010 Feb 24. [Epub ahead of print]*

Abstracts and oral presentations:

A summary of the above data has been presented in the following meetings:

1. Poster presentation: The Endocrine Society's meeting 89th Annual Meeting, Toronto, Canada, June 2007
2. Oral presentation: The 6th Annual Canadian Prostate Cancer-BioNet Meeting, Montreal, Canada, April 22-24, 2007
3. Poster presentation: The Endocrine Society's meeting 90th Annual Meeting, San Francisco, CA, June 2008
4. Oral presentation: The urologic science meeting, Vancouver, Canada, June 12, 2008
5. Poster presentation: the 4th PacRim Breast and Prostate Cancer, Whistler, Canada, August 2008
6. Oral presentation: Androgen Receptor Modulation by non-Androgenic Factors and Co-activator TAF1, Vancouver BC, Taylor Fidler Theater, February 2009
7. Chapter 4 of my PhD thesis, University of British Columbia, December 2009

CONCLUSION

The molecular mechanisms responsible for the development of castration-resistant prostate cancer are largely unknown, but typically they do not appear to involve the loss of AR expression (34). The up-regulation of AR-target genes and over-expression of AR at the protein and mRNA levels support the notion that AR activity is altered in castration-resistant states (35-38). There are a variety of molecular alterations that could lead to continued or amplified AR signaling following surgical or medical castration. Recent evidence suggests that AR-specific gene regulation may occur through interactions with unique coregulatory proteins. Since the N-terminus of AR (AR-NTD) is the least conserved, protein interactions in this region may dictate receptor-specific coregulation capacity.

Using the RTA yeast two-hybrid system TAF1 was identified as a novel AR-NTD-interacting protein (39) and this direct interaction is confirmed with full-length TAF1, using GST pull-down assays (Fig. 1C). Mapping of the TAF1 and AR interacting domains shows that the HAT and E1/E2 domains bind strongly to AR-NTD, mimicking the full length TAF1. The CTK domain

that was originally isolated by the RTA system interacts with all AR domains, but most strongly with the AR-DBD. In contrast, NTK does not have affinity for any AR domains, further indicating the specificity of these interactions (Fig. 1C & D). It has been reported by others that the N-terminus of TAF1 binds to the concave surface of TBP and consequently inhibits TBP/ TATA box contact, hence repressing transcription (21, 40). However, binding of activators, such as c-Jun with the N-terminus of TAF1 releases this inhibition, resulting in transcription initiation (41). Accordingly, the ability of TAF1 to interact with AR through multiple domains other than NTK suggests that TAF1 may play a role in modulating AR folding and one can speculate that, upon interaction with AR, the NTK release from the concave surface of TBP will initiate transcription. This hypothesis is supported by the fact that upon overexpression of TAF1 in both PC3 and LNCaP cells in the presence of nuclear AR (hormone induced activation), AR activity is increased (Fig. 2A&B), whereas siRNA knock down of TAF1 suppresses AR activity (Fig. 2C&D).

We also find that TAF1 expression levels were increased in prostate cancer patients who underwent NHT treatment for more than 3 months (Fig. 4), suggesting TAF1/AR interaction might be clinically relevant. Co-immunoprecipitation assays demonstrate that the interaction between AR and TAF1 occurs in the nuclear extracts of LNCaP and C4-2 cells in the presence of ligand-activated receptor (Fig. 5A). Although the AR seems to be more associated with TAF1 in LNCaP cells, this could be explained by the lower amount of the nuclear AR in C4-2 cells under this experimental condition (Fig. 5A, lane 4 versus lane 2). The association of TAF1 and AR in both androgen dependent and castration-resistant prostate cancer cells, plus the fact that TAF1 expression level is increased in patients undergoing androgen ablation therapy, suggests that TAF1 may interact with AR and enhance receptor activity even when there are low levels of circulating androgens. Since TAF1 is a component of the general transcription machinery within the TFIID complex and directly associates with AR to modulate AR activity, we explored whether TAF1 binds to the PSA promoter/enhancer. Using ChIP assays with LNCaP cells, we found that TAF1 is associated with AREs in the proximal/enhancer promoters of the PSA gene and this association is AR-dependent since knocking down AR would make the PCR products undetectable at the enhancer and significantly decrease them at the proximal promoter (Fig. 5B). This observation strongly suggests that TAF1 is a novel coactivator of AR that binds to the PSA enhancer through AR.

Since E1/E2 has the most profound effect on AR activity, we also sought to determine whether ubiquitination of AR can be increased as a consequence of TAF1 overexpression. Interestingly, in the presence of proteasome inhibitor and expression of His-ubiquitin, TAF1 enhances the total amount of ubiquitinated AR within a prostate cancer cell line (Fig. 6A, lane 8 versus 7). In addition, the E1/E2 domain alone is able to increase the total amount of ubiquitinated AR (Fig. 6B). This supports our transactivation data (Fig. 3B), in which the E1/E2 domain enhances AR activity more than 3-fold. Furthermore, TAF1 can ubiquitinate AR even in the absence of proteasome inhibitor within the cells (Fig. 6A, lane 4) and *in vitro* (Fig. 6C). Since the majority of TAF1-induced poly-ubiquitinated AR is accumulated after the proteasome inhibition and since poly-ubiquitinated AR is not functional (25, 42), there are at least three possible mechanisms that could explain how TAF1 enhances AR transcriptional activity. First, TAF1 can poly-ubiquitinate AR through lysine 48, causing proteasome degradation of AR

mainly through Mdm2 (Fig. 6A & B). This would induce AR turnover and consequently enhance AR transcriptional activity. Although we could not detect any significant changes of the AR expression level by TAF1 using cyclohexamide (Calbiochem) at different time points (data not shown), this remains to be further investigated. Second, TAF1 may induce AR poly-ubiquitination on other lysine sites, such as K6 or K27, as recently reported with the RNF6 protein and AR (43). This type of AR-poly-ubiquitination does not lead to AR degradation, as in the case of Mdm2. In contrast, it can enhance AR activity through modulation of AR binding proteins/chromatin, as has been shown with p53- and Met4-poly-ubiquitination (44, 45). These alternative mechanisms will be explored further in future studies. The third explanation could be that TAF1 may affect AR either through ubiquitination or phosphorylation of a cofactor(s), such as the possibility of the Mdm2 ubiquitination by TAF1 (Fig. 6A).

In conclusion, our results suggest that TAF1 is a coactivator of AR that binds and differentially enhances AR transcriptional activity most likely through ubiquitination of AR. Accordingly, an increase in TAF1 expression during NHT therapy for advanced prostate cancer, especially with treatment extended over 6 months, could be a compensatory mechanism adapted by cancer cells to overcome lack of circulating androgens.

Figures:

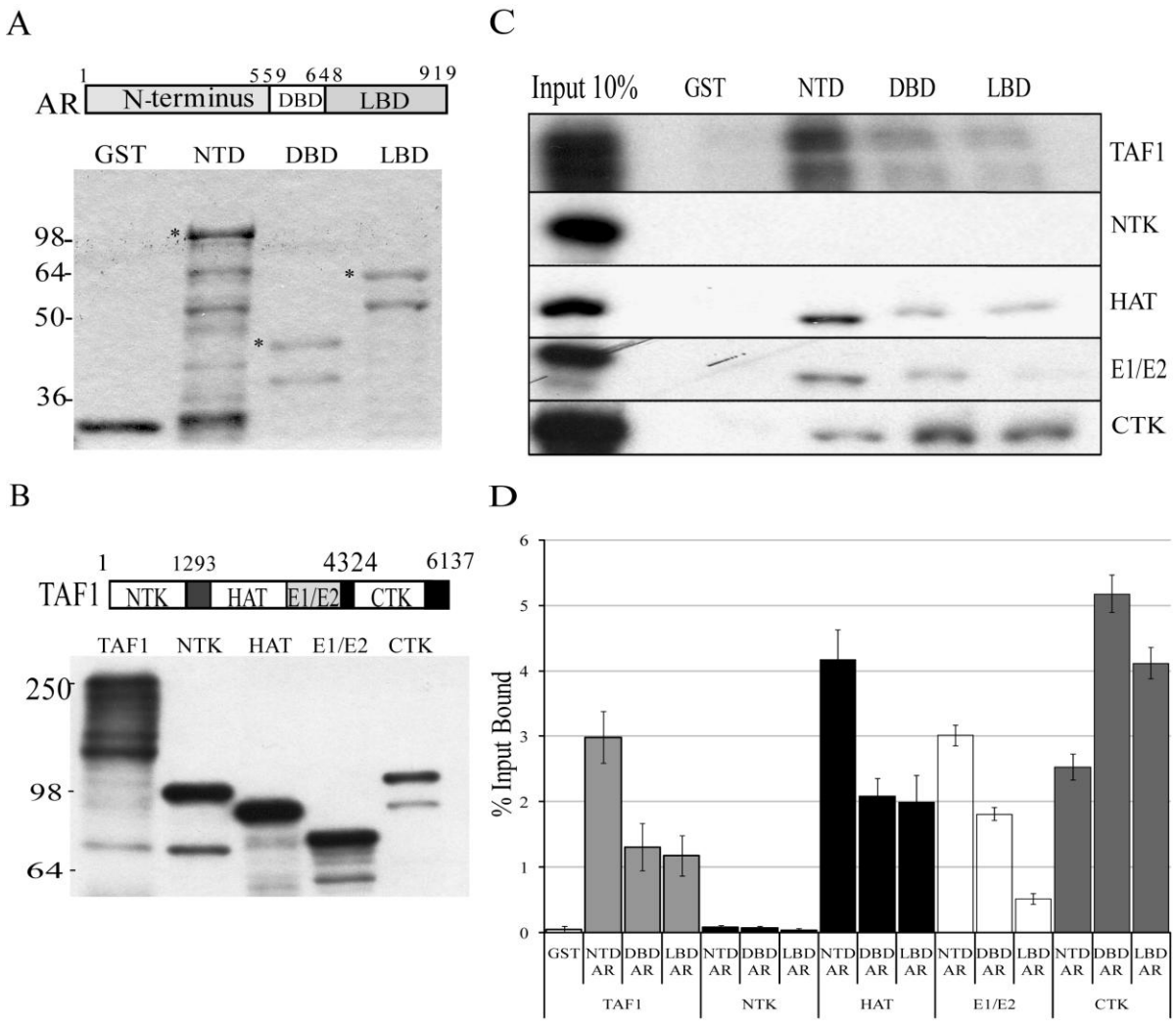


Figure 1. TAF1 binds AR through HAT and E1/E2 domains *in vitro*.
A. GST-fused AR domains (N-terminus =NTD, DNA binding domain =DBD, ligand binding domain =LBD) were expressed in E. Coli BL21 and purified using glutathione beads. Fusion protein-bound bead volumes were titrated, eluted with sample buffer and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. The eluent in each case was run alongside known amounts of BSA (ranging from 250 to 1000 ng) to generate a standard curve for protein concentrations. Equimolar amounts of non-degraded proteins (*) were used in GST pull-down assays. **B.** [^{35}S] Radiolabeled TAF1 and its domains (N-terminal kinase (NTK), Histone acetylation (HAT), Ubiquitin activating conjugating (E1/E2), and C-terminal kinase (CTK) were generated using *in vitro* Transcription/Translation kit. **C.** GST pull-down assay. Equivolume of [^{35}S] labeled TAF1, [^{35}S] labeled NTK, [^{35}S] labeled HAT, [^{35}S] labeled E1/E2, [^{35}S] labeled CTK were incubated with GST-AR fragments bound to agarose beads. GST alone coupled to agarose beads was used as negative control. **D.** Dried gels were also analyzed using a Phosphorimaging screen. Quantity One software was used to obtain data (counts/mm2) for radiolabelled protein bands. All pull-downs were done in triplicate, averaged, and normalized as a function of the percentage input bound.

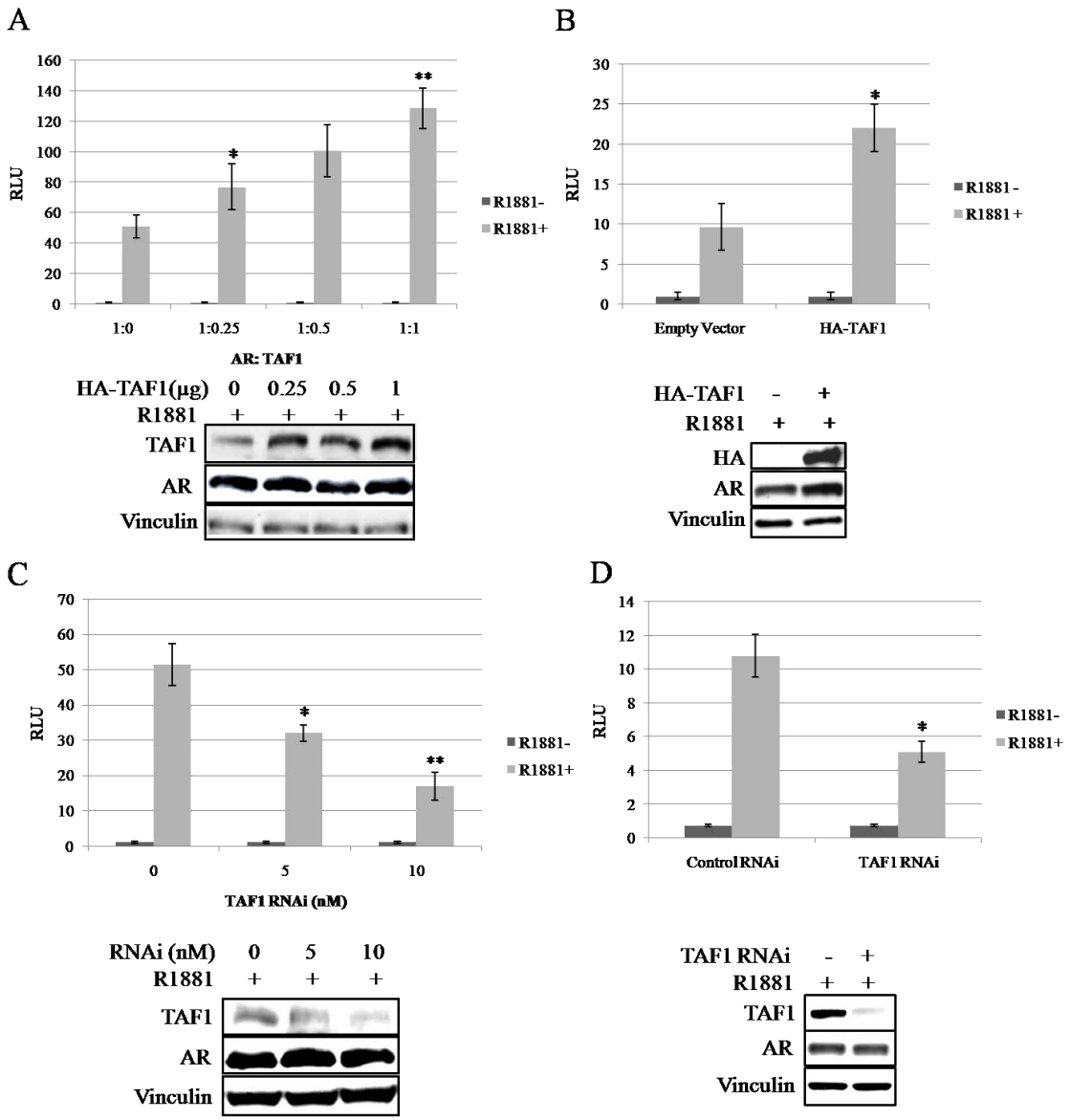


Figure 2. TAF1 modulates AR transactivation.
PC3 cells were co-transfected with 1.5 µg/well full length AR (pAR_6) and 0.2 µg/well pARR3-tk-Luc reporter, 0.1 µg/well pRLtk-renilla, and increasing amounts of pHA-TAF1 **(A)** or increasing amounts of TAF1 RNAi duplexes **(C)**. LNCaP cells were co-transfected with 1 µg/well pPSA-Luc, 0.1 µg/well pRLtk-renilla and 1 µg/well pCS2+HA-hTAF1 **(B)** or 10 nM TAF1 RNAi duplexes **(D)**. Transfected cells were growing in the presence or absence of 1 nM R1881 for 24 h (A, B) or 48 h (C & D) before harvesting for luciferase assay and Western blot analysis. Luciferase units (RLU) are expressed relative to protein values for each sample. All luciferase values are given as the mean (± SEM) of triplicate readings. Graphs are representative of the 3 independent experiments. * indicates a $p<0.05$ compared to empty vector control. ** indicates a $p<0.05$ compared to *.

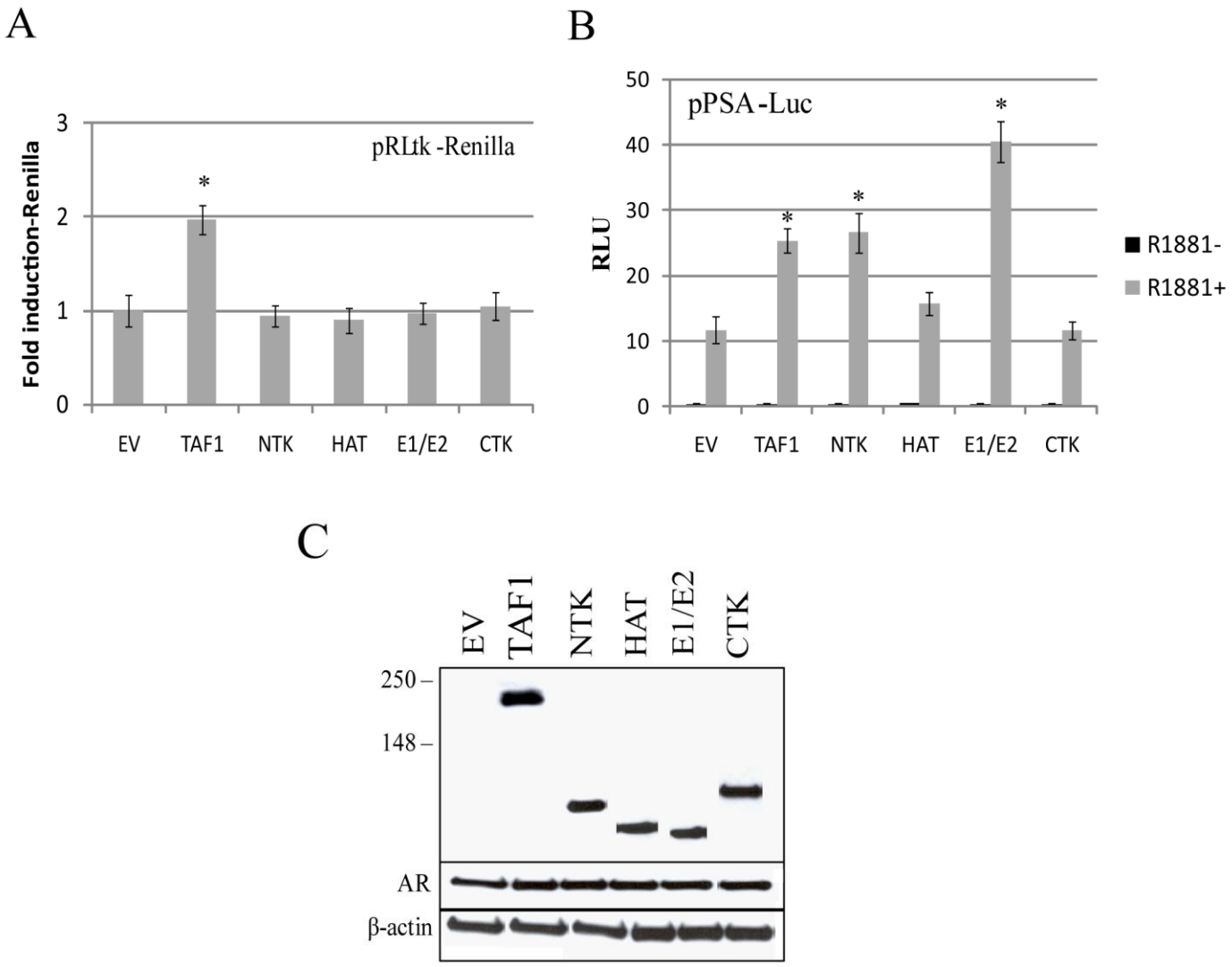
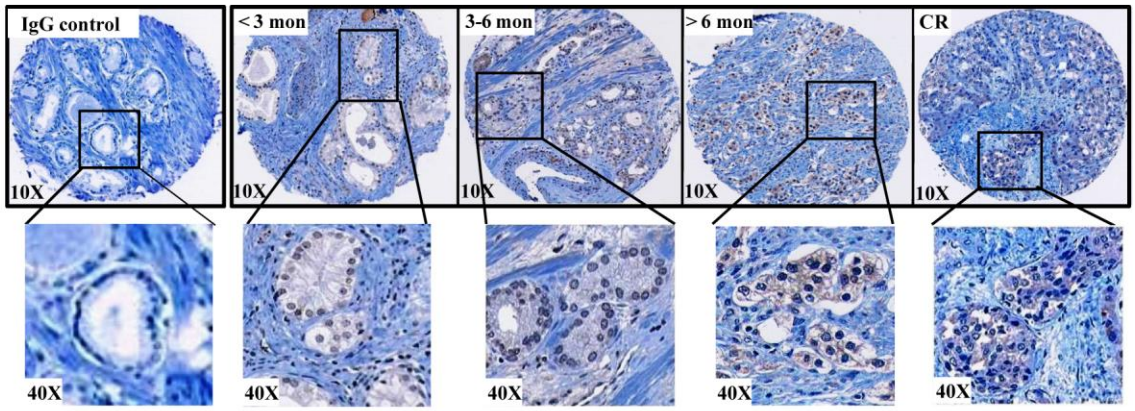


Figure 3. N-terminal kinase and ubiquitin activating/conjugating domains of TAF differentially enhance AR transactivation. LNCaP cells were co-transfected with either pHA-TAF1 or one of its four domains (pV5-NTK, pV5-HAT, V5-E1/E2, or pV5-CTK) (1 ug/well) and the pPSA-Luc and pRLtk-renilla. Transfected cells were growing in the presence or absence of 1 nM R1881 for 24 h before harvesting for luciferase assay. A) luciferase units were normalized to protein. B) fold-induction of renilla units were plotted against empty vevtor, TAF1 or its domains. * indicates a $p<0.05$ compared to empty vector control. C) Western blot analysis for AR, β-actin, empty vector (EV) (1), TAF1 (2), NTK (3), HAT (4), E1/E2 (5) and CTK (6).

A



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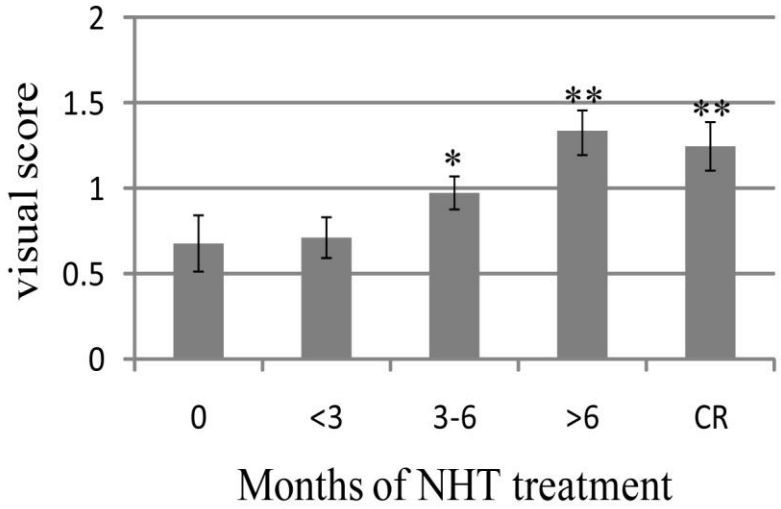

Figure 4. Tissue microarray analysis TAF1 expression in prostate cancer. *(A)* A NHT tissue microarray was stained with an antibody that recognizes TAF1 (abcam) or rabbit IgG (negative control). Staining intensity was scored from 0 to 3 by a pathologist. Slides were visualized under 10X magnification and further magnification (40X) of delineated areas is shown. *(B)* Visual score of samples with TAF1 staining intensity of 0–3 is given for each treatment group. * indicates that there is significant difference over 0 months. ** indicates that there is significant difference over 3-6 months.

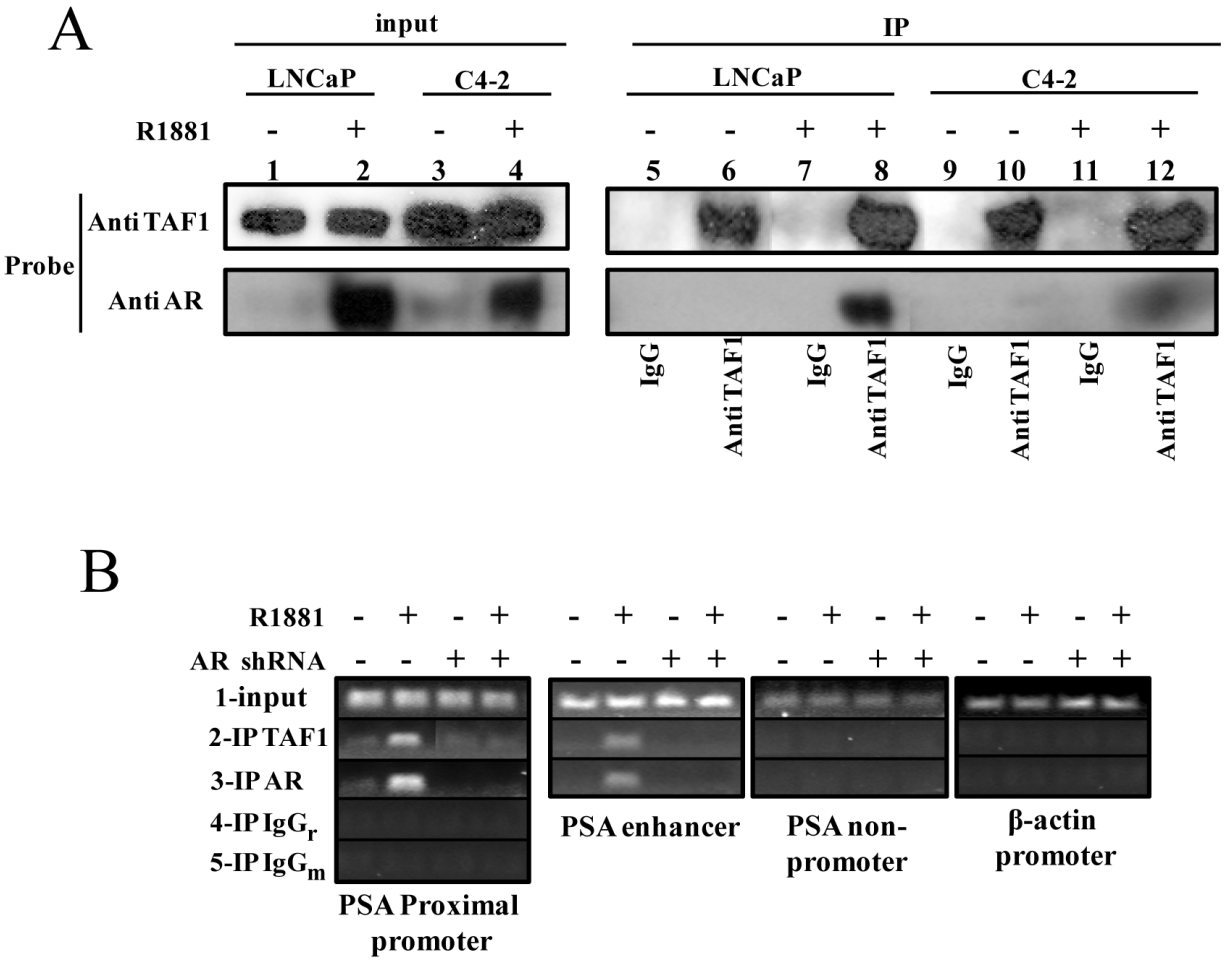

Figure 5. TAF1 interacts with AR within a prostate cancer cell line. *(A)* LNCaP or C4-2 cells were transiently transfected with HA-TAF1, grown in 5% charcoal stripped serum media (CSS) for 24 h and then treated ± 1 nM R1881 (synthetic androgen) for 4 h. Nuclear proteins were extracted and TAF1 was immunoprecipitated (IP) followed by Western blot analysis, probing with anti AR or TAF1 antibodies. *(B)* LN-shRNA$_{AR}$ or LN-shRNA$_{SC}$ cells were grown in CSS for 24 h and then treated with DOX for 48 h followed by 4 h ± 1 nM R1881 induction. Cells were then cross linked, DNA sheared and then the protein/DNA complexes were immunoprecipitated with anti TAF1 or anti AR and the promoter of the PSA proximal/enhancer were PCR amplified. The non-promoter region of PSA and β-actin primers were used as negative controls for TAF1 enriched protein/DNA complexes. IgG$_r$ = rabbit IgG, IgG$_m$ = mouse IgG.

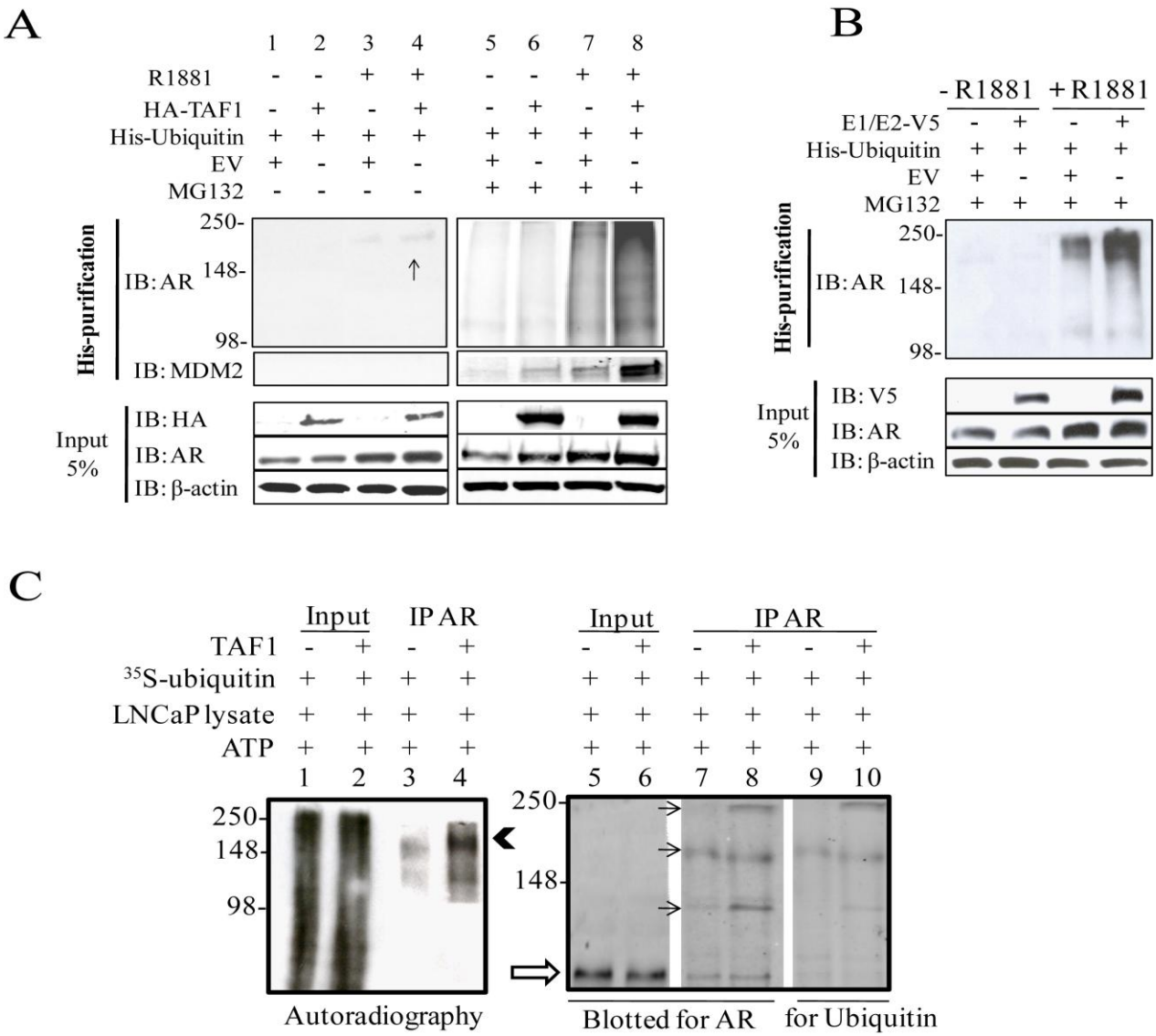

Figure 6. TAF1 ubiquitinates AR. *(A)* LNCaP cells were transfected with 2 µg pHis$_6$-ubiquitin and either 6 µg of pHA-TAF1 or empty vector. Cells were then treated with 5% CSS RPMI ± 1 nM R1881 followed by 10 µM MG132 or vehicle for 6h. After harvesting and lysing the cells in RIPA buffer, 5% of cell lysate was used as an input (lower panels) and the remainder was mixed with 50 µl Ni^{2+}-NTA-agarose beads. The mixture was rotated at 4ºC for 3 h and then affinity pulled down followed by Western blot analysis for AR and Mdm2 *(upper and middle panels)*. The input was blotted for HA, AR and β-actin. Arrow shows the poly-ubiquitinated AR in the absence of MG132 and when TAF1 is overexpressed *(B)* Experiments were designed as above, except cells were transfected with the pE1/E2 domain instead of the full length protein. *(C)* Nuclear extracts of HeLa cells were subjected to IP using antibody against TAF1 or IgG. LNCaP cell lysate was incubated with 1 mM ATP, ^{35}S-ubiquitin in the absence (lanes 1, 3, 5, 7 and 9) or presence of TAF1 IP (lanes 2, 4, 6, 8 and 10) in HEMG buffer. After 1 h incubation at room temperature, AR was immunoprecipitated followed by autoradiography (left panel) and Western blot for AR and ubiquitin (right panel). Lanes 1, 2, 5 and 6 = Input (5%) from LNCaP lysate before IP of AR. Solid arrow head and arrows show ubiquitin-conjugated AR. The open arrow shows non-ubiquitinated AR.

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